PATENT SPECIFICATION

563,651

Convention Dates (United States of America)

Dec. 5, 1941:
Dec. 5, 1941:

Corresponding Applications in United Kingdom

No. 17102/42. } dated Dec. 1, 1942.
No. 17103/42. }

(One Complete Specification left under Section 91 (2) of the Patents and Designs Acts, 1907 to 1942.)

Specification Accepted: Aug. 24, 1944.

(Under Section 6 (1) (a) of the Patents &c. (Emergency) Act, 1939, the proviso to Section 91 (4) of the Patents and Designs Acts, 1907 to 1942, became operative on Aug. 16, 1944).

COMPLETE SPECIFICATION

The Preparation of Physiologically Active and Antigenic Substances

We, THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA, a Corporation organized and existing under the laws of the State of Pennsylvania, United States of America, of 3446, Walnut Street, Philadelphia, Pennsylvania, United States

vaccines possess a high antigenic potency higher than that of virus vaccines for the respective diseases, that could be obtained by any of the heretofore known practical means, and, on administration, elicit the production of antibodies capable of

ERRATA

SPECIFICATION No. 563,651.

Page 2, line 108, *after* " infected " *insert* " with "
Page 3, line 9, *after* " which " *insert* " vaccine "
Page 3, line 24, *for* " Ilantoic " *read* " allantoic "
Page 3, line 95, *for* " ligt " *read* " light "

THE PATENT OFFICE,
November 18th, 1944.

animals, (for example, canine distemper, equine encephalomyelitis and the like) comprise the virus of such diseases, either the living virus, fully virulent or attenuated, or the inactivated virus, associated with a basic-protein-precipitant as a so-called basic-protein-precipitant-animal-infectious-disease virus complex or aggregate having a very exceedingly low percentage of sensitizing proteins incapable of eliciting antibody response to the virus, that is, proteins from the medium in which the virus was propagated and which upon injection might produce undesirable disagreeable reactions. These

[*Price* 1/-]

and allantoic fluids accompanying chick or other fowl embryos. The quantity of the basic-protein-precipitant need be only sufficient effectively to form, under the reaction conditions, the amount of complex that could be practically produced from the quantity of virus-containing material employed. Where necessary, the hydrogen ion concentration of the reaction mixture is adjusted to a point to permit the effective precipitation of the complex. The precipitated complex is separated by suitable means such as sedimentation preferably by centrifugal force followed by decantation, and, with or without subse-

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The Preparation of Physiologically Active and Antigenic Substances

We, THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA, a Corporation organized and existing under the laws of the State of Pennsylvania, United States of America, of 3446, Walnut Street, Philadelphia, Pennsylvania, United States of America, do hereby declare the nature of this invention and in what manner the same is to be performed, to be particularly described and ascertained in and by the following statement:—

This invention relates to virus vaccines that elicit in man and other animals the production of antibodies capable of neutralizing the virus of infectious diseases of man and other animals, which vaccines contain a complex broadly referred to as a basic-protein-precipitant-animal-infectious-disease virus complex and referred to in the appended claims as a precipitast/virus complex, and also relates to the complex and the preparation of it and of the vaccines.

I have found that advantageous virus vaccines effective in the prophylaxis of infectious virus diseases of man, (for example, influenza, yellow fever, rabies, St. Louis encephalitis and the like) and other animals, (for example, canine distemper, equine encephalomyelitis and the like) comprise the virus of such diseases, either the living virus fully virulent or attenuated, or the inactivated virus, associated with a basic-protein-precipitant as a so-called basic-protein-precipitant-animal-infectious-disease virus complex or aggregate having a very exceedingly low percentage of sensitizing proteins incapable of eliciting antibody response to the virus, that is, proteins from the medium in which the virus was propagated and which upon injection might produce undesirable disagreeable reactions. These vaccines possess a high antigenic potency higher than that of virus vaccines for the respective diseases, that could be obtained by any of the heretofore known practical means, and, on administration, elicit the production of antibodies capable of neutralizing the virus.

Likewise, the complex or aggregate may contain the fully active virus, or the attenuated or the inactivated virus.

The vaccines, as well as the complex or aggregate alone, may contain separately any of the types or strains, or any desired combination of such types or strains of a specific infectious disease virus, such as the influenza vaccines of type A or type B, or any desired combination thereof.

The method of preparing the complex or aggregate as well as the vaccines of the invention comprises the essential step of adding a suitable or the desired basic-protein-precipitant, in convenient form (e.g. solution, suspension, paste or powder) to a suitable starting material, preferably in liquid form, containing the desired virus in active, attenuated or inactivated state, for example, the separate or combined influenza virus infected amniotic and allantoic fluids accompanying chick or other fowl embryos. The quantity of the basic-protein-precipitant need be only sufficient effectively to form, under the reaction conditions, the amount of complex that could be practically produced from the quantity of virus-containing material employed. Where necessary, the hydrogen ion concentration of the reaction mixture is adjusted to a point to permit the effective precipitation of the complex. The precipitated complex is separated by suitable means such as sedimentation preferably by centrifugal force followed by decantation, and, with or without subse-

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quent washing with suitable washes, the complex may be preserved by suitable means, for example, by vacuum desiccation from the frozen state.

The vaccines may be put up directly from the complex at any of the described stages after its formation, or, depending on the starting virus-infected material, may involve suitable washing preferably with a solution that does not dissolve the complex, for example, a one per cent. aqueous solution of protamine or other basic-protein-precipitant after which the complex is advantageously re-suspended in a solution suitable for injection such as a buffered salt solution. Such re-suspension may be to any desired concentration and advantageously to any higher concentration than that of the virus in the original material in which it was propagated even up to ten times, or more, that concentration. If desired, there may be added to the vaccine any other desired agents such as a suitable preservative, for example, phenyl mercuric nitrate.

Among the suitable basic-protein-precipitants are included especially the basic proteins particularly those relatively simple, basic proteins obtainable from the sperm of fish, such as the protamines, as spermine, salmine, clupeine, scombrine, cyclopterine, sturine, salmiridine and the like; and also the histones exemplified by thymus histone and the like, and also the globins, for example, globin, the animal protein existing in hemoglobin. Also included are the basic degradation products of the protamines, such as result from hydrolysis progressively, as the protones, the polypeptides and the basic amino acids, exemplified by arginine, histidine, lysine and citrulline. The histone esters or hydrolysates, for example, the hydrolysates of histone obtained by boiling thymus histone in known manner with sulphuric acid and separating the hydrolysate by addition of alcohol, or the esters of histone obtained by introducing hydrogen chloride into a suspension of histone in methanol and adding ether to precipitate the hydrochloride of the ester are also included. Along with the polypeptides may be considered the decarboxylated derivatives thereof referred to as decarboxy-polypeptides, so that both polypeptides and decarboxy-polypeptides may be broadly termed polypeptide substances. Thus, the expression basic-protein-precipitant is used generically herein and in the claims to embrace broadly the precipitating agents, whether natural or synthetic, capable of precipitating the virus to form the desired complex or aggregate therewith, and it includes, only by way of example, the basic proteins and basic degradation products of proteins, given hereinabove as illustrative types.

The vaccines of the invention cover broadly those related generally to viruses that cause infectious disease in man and other animals, exemplified by, but not restricted to, the viruses of equine encephalomyelitis, yellow fever, rabies, St. Louis encephalitis, canine distemper, influenza and of other common virus diseases of man and other animals. The infected starting materials containing the desired virus may be of any of the known forms in which the virus may be propagated, for example, mouse brain emulsions as in rabies and influenza, horse brain emulsions as in equine encephalomyelitis, tissue cultures as in rabies and yellow fever, fowl embryo emulsions as in equine encephalomyelitis and fowl egg extra-embryonic fluids (e.g. allantoic and amniotic fluids) as in equine encephalomyelitis and influenza, some of which starting materials, if desired, may be subjected to any of the usual preliminary treatments to eliminate some undesirable components, as, for example, dialysis in the case of fowl egg extra-embryonic fluids to remove dialyzable substances and uric acid, or treatment to attenuate or inactivate the virus. In the latter case, inactivation may be accomplished by any convenient method, for example, physical such as heat, ultra-violet light and the like, or chemical such as with formalin.

The invention may be illustrated by, but not restricted to, the following examples.

EXAMPLE 1.

In spite of their low total protein content, the fluids of the allantoic sac (allantois) and the amniotic sac (amnion) accompanying chick embryos infected influenza virus, for example, of type A, contain large amounts of both the active influenza virus and the complement fixation antigen. Although these fluids, singly or combined, have a relatively small amount of cellular debris and while dialysis will eliminate therefrom the uric acid along with readily dialyzable substances, non-dialysable substances other than virus, for example egg proteins, remain in the fluids in relatively large amounts. To 100 cc. of untreated allantoic and amniotic fluids containing the influenza virus, is added about 20 cc. of an aqueous solution containing 10 mg. per cc. of spermine. As the pH is in the neighbourhood of 8.3 a precipitate appears immediately, which precipitate is the spermine-influenza virus complex or aggregate. The precipitate is separated by sedimentation preferably assisted by centrifuging the mixture, for example, in

the angle centrifuge at 5000 RPM. The supernatant liquid is drawn off. The precipitate (complex) is a yellow to creamy, fibrous, stringy mass, characterized by low solubility in water or salt solution. Upon resuspending the complex or aggregate in a buffered physiological saline solution to a volume of 100 cc., there is obtained a solution effective as a vaccine, which contains only about 0.4% of the nitrogen contained by the original infected egg fluid used as the starting material.

Used as a vaccine on mice, this solution of the spermine-influenza virus complex showed a protection equal to that exhibited by the whole extra-embryonic fluid (untreated infected allantoic and amniotic fluids) despite the fact that only a relatively small portion of the total egg fluid protein was present.

EXAMPLE 2.

Twenty cc. of a 1% aqueous solution of protamine is added to 100 cc. of clear extra-embryonic fluid (llantoic and amniotic fluids) containing influenza virus of type B. The pH being about 8.3, a precipitate appears immediately as evidenced by the turbidity showing up in the mixture. The precipitate is then sedimented by centrifugation and the supernatant liquid is decanted, leaving the precipitate which is the protamine-influenza virus complex or aggregate, having the same physical appearance as the aggregate of example 1. Upon re-suspending the precipitated complex in buffered salt solution to the original volume of 100 cc., the preparation shows approximately the same virus infectivity as the original infected fluid starting material. This buffered salt solution containing the complex is effective, after suitable attenuation or inactivation, as a vaccine.

EXAMPLE 3.

Clear extra-embryonic fluid containing the virus of type A, as in example 1, was first attenuated by the addition of one part of formalin per thousand and then treated with the protamine as in example 1. The pH was about 8.0 and the aggregate precipitated had the physical appearance of and was worked up in the same manner as that of example 1, the final product showing the same activity as that of example 1.

EXAMPLE 4.

Clear extra-embryonic fluid containing the virus of type A, as in example 1, was first attenuated by exposure to ultraviolet light for between seven to ten minutes and then treated with protamine as in example 1. The pH was about 8.0 and the aggregate precipitated had the physical appearance of and was worked up in the same manner as that of example 1, the final product showing the same activity as that of example 1.

The aggregate and the vaccine in each of the above examples possess complement fixation antigen.

While in the above examples the pH of the reaction mixture was about 8.3, in such cases where the pH is outside of the range of from about 7.0 to about 9.0, the pH should be adjusted to within that range in the case of precipitation with a protamine.

While the inactivation in example 3 was effected by using one part of formalin in one thousand parts of virus-containing starting material, the inactivation may be similarly carried out by using one part of formalin in from about five hundred to about ten thousand parts of virus-containing starting material. While the inactivation was carried out on the starting material as shown in examples 3 and 4, an avirulent complex or vaccine may also be obtained by using the active virus starting material as in examples 1 and 2 and carrying out the inactivation of the virus after the complex of examples 1 and 2 has been precipitated, by using any desired inactivating treatment, such as with ultra-violet ligt, or with a chemical such as formalin.

EXAMPLE 5.

In spite of their low total protein content, the fluids of the allantoic sac (allantois) and the amniotic sac (amnion) accompanying chick embryos infected with equine encephalomyelitis virus contain large amounts of the active virus. Although these fluids, singly or combined, have a relatively small amount of cellular debris and while dialysis will eliminate therefrom the uric acid along with readily dialyzable substances, non-dialyzable substances other than virus, for example, egg proteins, remain in the fluids in relatively large amounts. To 100 cc. of untreated allantoic and amniotic fluids containing the animal-infectious-disease, is added about 20 cc. of an aqueous solution containing 10 mg. per cc. of spermine. As the pH is in the neighbourhood of 8.0, a precipitate appears immediately, which precipitate is the spermine-equine encephalomyelitis virus complex or aggregate. The precipitate is separated by sedimentation preferably assisted by centrifuging the mixture, for example, in the angle centrifuge at 5000 RPM. The supernatant liquid is drawn off. The precipitate (complex) is a yellow to creamy, fibrous, stringy mass, characterized by low solubility in water or salt solutions. Upon re-suspending the complex or aggregate in a buffered physiological saline solution to a volume of 100 cc.,

there is obtained a solution effective, after suitable attenuation or inactivation, as a vaccine.

These examples are merely illustrative of the process of the invention as well as of the basic-protein-precipitant-animal-infectious-disease virus complex or aggregate and of the vaccines of the invention, of which the protamine-animal-infectious-disease virus complex vaccines are especially useful, such as the basic-protein-precipitant-influenza virus complex as the protamine-influenza virus complex vaccines.

While the above examples show the treatment of the infected extra-embryonic fluids directly with the basic-protein-precipitant without any dialysis, it is also possible to carry out the same process and to obtain the same complex and vaccines after the extra-embryonic fluid is first dialyzed to eliminate, for example, the uric acid. In either case, the aggregate may be rid of any occluded dialyzable substances by dialysis after suspending the separated aggregate in a minimum amount of suitable liquid.

While the starting material in the specific examples contained the active virus, starting material containing either the attenuated or inactivated form of the desired virus may be employed yielding with the latter an avirulent vaccine which can also be obtained if the product resulting from the use of an active virus, after the precipitation of the complex or aggregate, is inactivated at any subsequent step in the procedure by any convenient method as hereinabove noted.

Since the precipitated complex obtained in the process is readily re-suspended, whenever desired, the complex may be washed one or more times with a liquid in which it does not dissolve, such as an aqueous 1% protamine solution or similar solution of any other basic-protein-precipitant.

While the invention has been illustrated by certain specific embodiments thereof, it is understood that certain substitutions or modifications can be made therein, for in place of the specific type or strain of virus or the specific basic-protein-precipitant used in the examples, any other suitable type or strain of the virus or species or type of basic-protein-precipitant or other infected starting material may be employed according to the invention which is intended to be limited to the available scope of the appended claims.

Having now particularly described and ascertained the nature of our said invention and in what manner the same is to be performed, we declare that what we claim is:—

1. The method of preparing a precipitant/virus complex which includes the step of mixing a basic-protein-precipitant as hereinbefore defined with a starting material containing the desired virus in active, attenuated or inactivated state to form the precipitant/virus complex.

2. A method as claimed in Claim 1 wherein the precipitant/virus complex is subsequently separated from the reaction mixture.

3. A method as claimed in Claim 1 or Claim 2 wherein the basic-protein-precipitant is employed in the form of a solution, suspension, paste or powder.

4. A method as claimed in any one of the preceding claims wherein the virus-containing material is employed in liquid form.

5. A method as claimed in any one of the preceding claims wherein the hydrogen in concentration of the reaction mixture is adjusted to a point to permit of effective precipitation of the complex.

6. A method as claimed in any one of the preceding claims wherein the precipitated complex is separated by sedimentation e.g. by centrifugal force followed by decantation.

7. A method as claimed in any one of the preceding claims wherein the separated complex is washed e.g. with a dilute aqueous solution of protamine or other basic-protein-precipitant.

8. A method as claimed in any one of the preceding claims wherein the complex is subjected to desiccation under such conditions that the complex retains its capacity of eliciting the production of antibodies capable of neutralising the virus.

9. A method as claimed in Claim 8 wherein the complex is subjected to vacuum desiccation from the frozen state.

10. A method as claimed in any one of the preceding claims wherein the basic-protein-precipitant consists of a protamine, an histone or a globin or a basic degradation product of a protamine e.g. a protone, a polypeptide or a basic amino acid.

11. A method as claimed in any one of the preceding claims wherein the virus is that of influenza, yellow fever, rabies, equine encephalomyelitis.

12. A method as claimed in Claim 10 wherein the influenza virus employed consists of type A or of type B or of mixtures of type A and type B.

13. A method as claimed in any one of the preceding claims wherein the precipitant/virus complex is formed by mixing the basic-protein-precipitant with infected egg extra-embryonic fluids e.g. with infected fowl egg extra-embryonic fluids.

14. A method of preparing a precipitant/virus complex substantially as described in any one of the specific examples hereinbefore set forth.

15. A precipitant/virus complex whenever prepared or produced by the method claimed in any one of the preceding claims.

16. A method of preparing a virus vaccine which comprises suspending a complex as claimed in Claim 15 in a liquid medium suitable for injection.

17. A virus vaccine whenever prepared or produced by the method claimed in Claim 16.

Dated this 1st day of December, 1942.

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